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CELLULAR AND MITOCHONDRIAL DYNAMICS IN CHINESE HAMSTER CELLS

Dennis Warren Ross
(Ph.D. Thesis)

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CELLULAR AND MITOCHONDRIAL DYNAMICS
IN CHINESE HAMSTER CELLS

Dennis Warren Ross

ABSTRACT

The mammalian cell division cycle encompasses a complicated series of events involving the orderly increase in all subcellular components necessary for a parent cell to divide into two daughter cells. To elucidate some of these steps, a study was undertaken to measure the quantitative increase in whole cell volume as a cell grows and divides, and the apportionment of this cell volume into nuclear, cytoplasmic and mitochondrial compartments. A continuously proliferating cell culture of Chinese hamster fibroblasts, V79-S171 cells was used. Changes in cell cycle compartment dynamics were simultaneously measured to allow for the correlation of morphological and metabolic data. Electronic volume sizing, electron microscopy, radioactive labeling with autoradiography and cytology were employed. Cell populations were synchronized by mitotic selection.

Initial experiments demonstrated that volume sizing using a Coulter type aperture with appropriate electronic amplification and analog to digital conversion with pulse height analysis, resulted in an accurate volume spectrum of the cell population, comparable with that from optical microscopic techniques. Subsequent volume sizing experiments in a synchronized cell population showed that cell volume increases in a smooth fashion as a
function of time. That is, no discontinuities in rate were
detectable at periods when cells may have been undergoing
metabolic shifts as, for example, through the phases of the cell
cycle associated with DNA synthesis, G₁, S, G₂.

The experimental data on cell volume changes in synchro-
nized population were found to agree most closely with an exponen-
tial mode of volume increase although a linear increase could not
be ruled out. Electron micrographic studies of the apportionment
of the volume of the growing cell amongst the nucleus and mito-
chondria revealed that nuclear volume changes parallel those of
the cell but that mitochondrial volumes do not. Mean total mito-
chondrial volume and number of mitochondria per cell showed a
significant decrease in G₁ and G₂ as compared to mid S. Absolute
mean values per cell for log phase Chinese hamster cells were
also directly determined, as follows: whole cell volume, 710 μ³;
nuclear volume, 190 μ³; total mitochondrial volume, 32 μ³; number
of mitochondria per cell, 60. These results are not consistent
with a model of simple doubling of mitochondria either synchro-
nously with the cell division cycle or asynchronously. Other
possible patterns of mitochondrial biogenesis are discussed.

Cell volume changes were also studied in a population with
limited proliferation potential--achieved by allowing a cell
culture to enter stationary phase. In order to measure the
nutrient depletion of cell culture medium the term nutritive
capacity was formulated to allow for an accurate prediction of
the onset and development of stationary phase. Biochemical and
cytologic studies were done to determine where in the cell cycle
cells came to rest when they ceased proliferation. It was found that these cells are initially arrested in the $G_1$ and $G_2$ compartments, but with time the $G_1$ compartment predominates. The exact kinetics of this redistribution was found to be dependent in a complicated way on the nature of the cell medium. Cell volume was found not to be tightly coupled to the metabolic state of the cell during stationary phase, in contradistinction to the coupling found in log phase. Cell volume dispersion of the population was in fact greatly increased in stationary phase.
ACKNOWLEDGEMENTS

The help, advice and concern of many people have been ingredients of this thesis and I should like to acknowledge them. Professor Warren K. Sinclair, Director, Division of Biological and Medical Research, Argonne National Laboratory, introduced me to cellular biophysics and started me in my investigation. He has been my co-author in the publication of a portion of the work presented here. The techniques of mammalian cell culture described in this report were learned in his laboratory.

Professor Howard C. Mel, Donner Laboratory, my research advisor and thesis committee chairman was intimately involved during the continuing progress of my research. In his laboratory and his office were these experiments and data continually reviewed. Helpful suggestions and an open forum for discussion were always available; he has given much direction to my career. Professors Robert Mortimer and H. John Burki with their attentions and advice, helped provide the scientific environment at Donner Laboratory which I enjoyed during this study. Technical assistance from the non-academic staff was also of great help with many specific phases of this research: at Argonne, Miss Grace Racster and Mr. Mel Long; at Donner, Mrs. Frances Taylor and Mr. Stephen Akeson. Lastly I would like to thank my family--my parents, Mr. and Mrs. Sol Ross and my wife Mrs. Sarah Ross for their loving support during my studies.
GENERAL INTRODUCTION

The growth and replication of cells is one of the fundamental processes of living systems. The cell's ability to grow and duplicate itself using organic materials supplied from its environment is the living response that bridges our understanding of the cell as a collection of macromolecules and our observation of it as a living system. The problem has been studied at many levels in almost every biological system. To the biophysicist, the problem of cell growth and division begs for expression in terms of quantitative measures of rates of processes and their interactions in time. Mathematical models of cell growth are developed to correlate quantitative experimental information, to suggest new patterns of cell growth and to expand the understanding of the cell as a dynamic system.

The present study is an investigation of cell growth and division in mammalian cell cultures of isolated Chinese hamster lung fibroblasts. The emphasis is on quantitative measures of growth with mathematical modeling of observed behavior. Mammalian cells are technically more difficult to maintain and study in the laboratory than microbial populations, but their more complex cellular structure makes for a richer field of observation and is more relevant to abnormal growth processes such as the uncontrolled cellular growth that occurs in the disease state of cancer.

In cultures of Chinese hamster cells, population doubling occurs about every ten hours in the logarithmic phase of growth under optimal culture conditions. When there is no cell loss,
this time is equivalent to the cell generation time—the average interval between cell divisions. The population growth dynamics alone have in the past provided much information concerning cell growth. Observations of the distribution of cell generation times in yeast and hypotheses concerning cell growth were reviewed by Tobias in his article 'Quantitative Approaches to the Cell Division Process' (1) -- a paper which was instrumental in motivating the present study. Kubitschek has presented a similar discussion for bacteria (2,3).

The cell division cycle has been further subdivided into four compartments: M, G\(_1\), S, G\(_2\). This subdivision is based on the observable cytokinetics occurring during mitosis, M, and the biochemical activity of DNA synthesis occurring during S. The gaps between these events are denoted G\(_1\) and G\(_2\), respectively. These concepts are reviewed in an article by Nachtwey and Cameron, 'Cell Cycle Analysis' (4) and also by Mitchison, 'Markers in the Cell Cycle' (5). Cell growth in this thesis is considered in terms of cell volume changes during the cell generation cycle, and the apportionment of these changes in cell volume amongst the nucleus, the mitochondria and the cytoplasm. The volume changes and distribution of cells in the cell cycle have also been studied in non-proliferating stationary phase cultures.

For organizational purposes the body of this thesis is divided into three main chapters:

I. Cell Cycle Volume Dynamics—Modes of growth in mammalian cells
II. Subcellular Volume Kinetics--Growth dynamics of mitochondria in synchronized Chinese hamster cells

III. Stationary Phase Cell Cycle Distribution--Cell cycle compartment analysis of Chinese hamster cells in stationary phase cultures

Each chapter will be introduced and discussed independently with a presentation of the appropriate materials and methods.
I. CELL CYCLE VOLUME DYNAMICS

INTRODUCTION

A number of recent investigations have dealt with the mode of cell growth during the generation cycle in single cells of various species (6-9). In bacteria, exponential increase in accumulation of macromolecules (DNA, RNA and protein) has been observed (6). In another conflicting study volume increase appeared to follow a linear mode (9). More complicated growth patterns may exist in cells in which differences in rates of macromolecular syntheses occur during the cell cycle. These apparently occur in bacteria (10) but the times involved are so short that the detection of volume changes concurrent with metabolic shifts, if these occur, would be extremely difficult. In mammalian cells (and in some other species also, e.g. (11)) changes occur, especially in DNA synthesis (12-14), which may be sufficiently discrete in time to allow detection of concurrent volume changes. In some instances complex patterns of volume increase have been observed (12,14,15). However, in another study (16), in mouse L cells, dry mass was observed to increase smoothly, but experimental accuracy was insufficient to identify the growth as either linear or exponential. The mass increase was, however, correlated with increasing RNA content in the cell.

In a preliminary report (17) from Warren Sinclair's laboratory several years ago, it was shown that the average volume of a synchronous population of Chinese hamster cells, grown in vitro, increased steadily with time during the cell generation cycle.
A comparatively simple mode of growth was indicated, exhibiting no discontinuities related to metabolic changes in the cell, such as onset or cessation of DNA synthesis.

Koch (18) has discussed the theoretical limitations of considering modal volume as a function of time and he suggests that the rate of volume increase, \( dV/dt \), is a more sensitive test of growth laws. Bell (19) and Bell and Anderson (7) have formulated a model of cell populations which uses rate of volume increase as a population parameter. The merits of this approach have recently been discussed by Anderson et al. (20). However this model, which has yielded much useful information concerning the interrelationship between the volume spectrum and growth parameters has not revealed definitive information on the nature of growth laws when applied to the asynchronous volume spectrum in particular cases (8,20). One objection to this method is that the rate of volume increase is not a directly measurable quantity and must be calculated from higher order moments of the volume spectrum and from other experimental data. On the other hand, average or modal volume and volume distribution width during the cell cycle are directly observable quantities not dependent on a subsequent analysis of cell population characteristics. However the growth parameter of principal interest, \( d\bar{V}/dt \), must still be derived from the average volume. At the present state of our knowledge presumably both approaches can be exploited to yield useful information.

The present chapter discusses the principal features of cell growth as observed in synchronous cultures of two sublines
of Chinese hamster cells. A high resolution apparatus involving a Coulter counter aperture and a 400 channel analyzer was used to measure the peak (modal) volume and the volume distribution halfwidth at half maximum height, as a function of time in the cell cycle. The resulting experimental data was compared with a mathematical model of cell volume increase which predicts the time development of the mean volume in a synchronous population with a small spread in cell doubling times.

MATHEMATICAL THEORY OF CELL GROWTH AND DIVISION IN SYNCHRONOUS POPULATIONS

A single cell increases its volume within a generation time until the initial volume is approximately doubled. After division its daughters repeat this cycle. A synchronous population of cells exhibits the same behavior in terms of the mean cell volume and the mean generation time. As synchrony decays due to the dispersion of generation times within a population of cells, the cyclic variation of the mean volume becomes damped and the volume approaches a constant average value. The cell number then approaches an exponential increase characteristic of a log phase, steady-state population. The aim here is to provide a model which will predict the time development of cell number and volume of a synchronous population. The model presented is the simplest form that was found convenient for comparison with experiment. The expression to be derived here for the increase of cell number with time can also be derived from more general expressions of Harris (21) and equation 21 of Rubinow (22),
which describe the number of cells in a given generation and then sum over all generations present in the population. The method employed in this chapter is quite applicable to situations in which the coefficient of variation of the distribution of generation times in the population is small and attention is confined to the first few generations so that all cell division takes place between the limits given by equation (1) below. If this condition does not apply (e.g. in later generations) the more general equations must be employed.

Cell Number

Assume that at time \( t = 0 \), we have a population of cells all of the same size \( V_0 \), and all of the same age (just divided). For a time no cells will divide, but as the average generation time of the population \( T_0 \) is approached, a fraction of the cells will divide within any given time interval. Define the \( n^{\text{th}} \) interval of division as:

\[
(n - 1/2) T_0 \leq t \leq (n + 1/2) T_0
\]

This is a time period which extends from the middle of the \( n^{\text{th}} \) generation cycle to the middle of the \( (n + 1)^{\text{th}} \) generation cycle.

Then the number of cells that will be found in the \( n^{\text{th}} \) interval of division is:

\[
N_n(t) = N_n^o \left[ 2 \int_0^t f_n(T) dT + \int_t^\infty f_n(T) dT \right]
\]

where \( N_n^o \) is the number of cells at the start of the interval.

\[
N_n^o = 2^{(n-1)} N_o
\]
$N_0$ is the number of cells at time $t = 0$ and $f_n(T)$ is a distribution function of cell generation times $T$, about a mean generation time $T_0$, given explicitly in equation (3).

Equation 2 has been derived directly from considerations of a synchronous population and its performance in subsequent generations. A different form of this expression, viz.

$$N_n(t) = N_0 n \left[ 1 + \int_0^t f_n(T) dT \right]$$

is more often used for populations in general when the number of cells present is obtained by summing over all generations.

Equation 2 is valid only if there is no overlap of generations between $(n - 1/2)T_0$ and $(n + 1/2)T_0$. This is the case in the experimental situations examined in this chapter.

The distribution function $f_n(T)$ describes the distribution of cells dividing with time during the $n^{th}$ generation. [In the first generation this corresponds to the distribution of generation times.]

$$f_n(T) = \left( \frac{2}{\pi n \sigma_1^2} \right)^{1/2} \exp \left( -\frac{1}{2} \left( \frac{T - nT_0}{n^{1/2} \sigma_1} \right)^2 \right)$$

Assume that the distribution of generation times is normal about a mean value, $T_0$, and that the distribution width increases according to the relation $\sigma_n$ (for the $n^{th}$ generation) = $n^{1/2} \sigma_1$. Thus, $f_n(T)$ describes the frequency of cells dividing in any given time interval for an initially synchronous population. As $n$ becomes large, the number of cells dividing in any time period approaches a constant times the number of cells present and the
increase in cell number becomes exponential with time.

There is some difference of opinion as to whether the distribution of generation times $f(T)$ or generation rates $f(1/T)$ is gaussian in an asynchronous population (1, 2). Assuming the generation times to be distributed normally implies that the distribution of generation rates is skewed towards slower rates. Similarly, assuming the rates to be gaussian means that the distribution of generation times is skewed towards longer times. This effect was investigated in the function $f_n(T)$ with an initial coefficient of variation (CV) = $\sigma_1/T_0 = 10\%$. The amount of skewness of either distribution was found to have a less than 1% effect on the predictions of this theory.

The initial behavior of the synchronous population is incorporated into the parameters $T_0$, the average generation time, and $\sigma_1$, the initial distribution width of $f_n(T)$ for $n = 1$. The effective distribution width in the $n^{th}$ cycle, $\sigma_n = n^{1/2}\sigma_1$ describes the decay of synchrony.

**Mean cell volume**

If the volume of a single cell can be written as a function of time $t$, and its generation time $T$, one can calculate the mean volume of the population by averaging over the distribution of interdivision times $f_n(T)$.

$$V_n(t) = \frac{2^{n-1}N_0}{N_n(t)} \left\{ 2 \int_0^t f_n(T) V_n(t,T) \, dT + \int_t^\infty f_n(T) V_n(t,T) \, dT \right\}$$

(4)
The factor of two in front of the first integral is due to the doubling of all cells with intervals of division $T \leq t$. The normalization factor is just the number of cells in the $n$th interval as a function of time.

We now require an expression for $V_n(t, T)$:

For linear volume increase,

$$V_1(t, T) = V_o (1 + t/T_o)$$
for $0 < t < T$;

$$V_2(t, T) = \frac{1}{2} V_o (1 + T/T_o) + V_o \left( \frac{t - T}{T_o} \right)$$
for $T < t < 2T$;

$$V_3(t, T) = \frac{1}{4} V_o (1 + T/T_o) + \frac{1}{2} V_o \left( \frac{T}{T_o} \right) + V_o \left( \frac{t - 2T}{T_o} \right)$$
for $2T < t < 3T$.  
(5)

For exponential volume increase,

$$V_1(t, T) = V_o \exp \left( \frac{(\ln 2) t}{T_o} \right)$$
for $0 < t < T$;

$$V_2(t, T) = \frac{1}{2} V_o \exp \left[ (\ln 2) \frac{T}{T_o} \right] \exp \left( \frac{(\ln 2) t - T}{T_o} \right)$$
for $T < t < 2T$;

$$V_3(t, T) = \frac{1}{4} V_o \exp \left[ (\ln 2) \frac{T}{T_o} \right] \exp \left( \frac{(\ln 2) t - 2T}{T_o} \right)$$
for $2T < t < 3T$.  
(6)

These sets of equations, (5) and (6) are merely cyclic ramps with either a linear or an exponential slope. We shall assume
(a) that different cells grow to different maximum volumes depending on their generation time $T$, i.e., control of division is independent of volume, and (b) that parent cells divide into two daughters each with one-half the parent cell volume (cf. reference 20). Then, the initial volume of the cell after division is treated as a function of the generation time within that cycle only. Biologically, one may expect that the volume of a cell is related to more of its past history, including all of its past generation times; however, it was not necessary to include these other variables in this model to obtain an accurate prediction of population behavior.

A more complete description of the volume increase of a population of cells would include, in addition to the distribution of generation times, the possibility of a distribution of rates, rather than the constant rate $1/T_o$. Calculations have been performed including these other factors, but they indicate that more detailed theory does not greatly add to the analysis of the present results.

When the analytic expressions for cell growth (equations (5) and (6)) are evaluated in the formulas (2) and (4), the parameters of cell number and mean cell volume can be plotted as continuous functions of time. The calculation requires numerical integration, which was performed on a digital computer. Figure 1 is a plot of these functions for a cell line with a generation time of 9.5 hours. Figure 1a shows the increase in cell number, which Engelberg has described as the "carpeted staircase function" (23). In Figure 1b is shown a plot of mean volume as a function of time.
assuming linear growth. The maximum mean volume does not reach $2V_0$ and the new minimum mean volume after division is greater than $V_0$. This "rounding" of the curve of mean cell volume vs. time is inherent in equation (4) and becomes progressively greater in successive cycles. Figure 1b shows clearly that even if growth is truly linear, "rounding" occurs because of the spread of generation times, and that this also reduces the length of the linear portion of growth in the next cycle. With data of coarse precision this effect may even give the appearance of other modes of growth (e.g. exponential). This fact, which was pointed out by W. K. Sinclair, has been used to help explain the shape of the growth curve in synchronous E. coli (9).

MATERIALS AND METHODS

Cell lines and growth conditions

The cells used were sublines of the V79 line of Chinese hamster cells which was originally grown from female lung tissue by Ford and Yerganian (24). One subline, designated V79-S171, has a generation time of 9.5 hours, subdivided into a pre-DNA synthetic period, $G_1$, of $\sim$1.5 h, a DNA synthetic period, $S$, of $\sim$6 h, a post-DNA synthetic period, $G_2$, of $\sim$1.5 h and mitosis lasted 0.5-1 h. The other subline, designated V79-325, had a longer $G_1$, $\sim$3.5 h and $S$, $\sim$9 h, while $G_2$ was about 1.5 h. Mitosis presumably lasted about 0.5-1 h also. Cells were grown in monolayer cultures attached to the surface of plastic dishes at 37°C in EM-15 medium (formulation given in Table 1) in a humid atmosphere of 2% CO₂ and air.
Synchronous cultures

Synchronous cells were obtained from asynchronous cultures in log phase by selection for poorly attached dividing cells using a standard shake-off procedure (25, 26). Aliquots of the resulting suspension of synchronous cells were inoculated into a series of dishes and incubated at 37°C. This temperature in the dishes was maintained constant to ± 0.1°C throughout the experiment. At half-hour intervals a dish was removed and cells were detached from the surface by treatment with trypsin (0.03%). Cells of this suspension were counted and their volume distribution was determined, in experiments at Argonne National Laboratory, by passing the cells through a 100 μ x 100 μ Coulter Counter aperture, the signals from which were fed through amplification stages to a 400 channel analyzer. An X-Y plotter was used to obtain immediate visual results of the volume distribution. In the Donner Laboratory experiments (University of California, Berkeley) the volume sizing equipment consisted of a 100 micron aperture (Particle Data, Franklin Park, Illinois) with amplification and accessory electronics designed by the Electronics Group at Donner Laboratory, and pulse height analysis via a PDP-8/I computer on-line (Digital Equipment Co., Maynard, Mass.).

Another set of culture dishes was used at less frequent intervals to establish the subdivisions of the cell generation cycle (24). This was accomplished by pulse labeling the cells with tritiated thymidine ($^{3}$$T$dr) for 15 min (0.1 μCi/ml, 14 Ci/mM) followed by autoradiography. As a check on the quality of synchrony, mitotic index, Blumenthal-Zaler index applied to
the first half cycle (27), and L index (26), were used. Typical values of these parameters for V79-325 cells were 77%, 95%, and 82%, respectively, as shown in Figure 5.

Electronic volume measurements

Two methods were used to calibrate the cell volume spectrometers. A photographic study of cell size was performed on 700 cells of an asynchronous population by measuring diameters of trypsinized cells under an optical magnification of 1800. This volume distribution is then compared to a volume distribution of 500,000 cells from the same population sized electronically, as shown in Figure 2. The high degree of correlation achieved (Chi-squared test shows that, to a greater than 95% confidence level, they are identical) provides a means of relating channel number to absolute volume in cubic microns. In addition, a reference distribution of latex spheres in the same range of volumes as the cells (7 to 14 μ in diameter) was checked every few hours to monitor the stability of the system.

A similar population of log phase V79-S171 cells was sized at Berkeley on the volume sizing equipment previously described for Donner Laboratory. Figure 3 shows the composite results for all three spectra: a. the histogram corresponds to the optical sizing; b. the solid curve is the volume spectrum taken at Argonne; and c. the dotted curve is the volume spectrum taken at Berkeley. The vertical scale of each curve has been displaced slightly to allow for comparison of their shapes. The curves obtained on the equipment at Argonne and the equipment at
Berkeley for the same cell line grown under identical conditions are equivalent (as tested by a comparison of the first three moments of the two normalized distribution) to a high degree of significance. The results shown in Figures 4, 5, 6 and 7 were obtained on the Argonne equipment, however identical results were obtained in Berkeley as well.

The possible effect of the size of the aperture on the volume distribution was checked at the suggestion of H. E. Kubitschek. In experiments reported herein an aperture 100 μ in diameter and 100 μ long was used. Similar results for the peaks of both the reference distributions and asynchronous distributions of cells were observed with an aperture of 85 μ x 185 μ, lent to W. K. Sinclair and myself by Kubitschek. This longer aperture permits greater transit times, which however appeared to be unnecessary in this instance because our analyzer was adjusted to obtain faster electronic response times. Coincidence counts (two cells simultaneously in the aperture) were nearly eliminated by the speed of the sizing system. A delay-monostable gate was set at 10 μs to hold the flow of information through the system open for only that length of time necessary for the full volume of the cell to be recorded.

The contents of the 400-word memory were presented to an X-Y plotter which had a null-balance feature to assure accurate plotting. Six typical distributions obtained from a synchronous population during the first two cycles of division are shown in Figure 4. Arrows above the volume spectra indicate the modal volume of the population. Three of the frames (a), (d), and (f)
show spectra at times when the population was undergoing cell division. For these distributions it was possible to separate the population into two subgroups; those cells about to divide and those cells which have recently divided. The arrows over the spectra in these frames indicate the modal volumes of each of these two subgroups and also of the population taken as a whole. The modal volume for the whole population is the weighted mean of the bimodal volume, with the weight factor being the fractional number of cells in each mode. Note: the model actually predicts the mean volume of the whole population rather than the peak or modal value. However, the mean and the mode were nearly identical for these distributions and the peak volume was considered to be the better parameter since it is less sensitive to noise and shape factors.

The resolution of the volume measurement performed by this electronic technique was about 1% (+ 1 channel number). In terms of absolute units, a measurement of an average cell of volume $710 \mu^3$ had an uncertainty of $\pm 25 \mu^3$, including the uncertainty in calibration. A method of increasing the precision would be, in theory, to spread the volume distribution over more channels of the analyzer, narrowing the width of the window on any one channel. This procedure also serves in our apparatus to introduce more statistical noise in the number of counts in any one channel for the number of cells available (about 5000 cells/spectrum) and there is no net gain in accuracy. In fact 200 channels was found to be an optimal spread of the volume distribution for this population of cell sizes. The electronic sizing technique was
limited to a certain resolution, probably about 1%, by the nature of the conversion of cell sizes to current pulses inside the Coulter aperture. The system would not respond in a reproducible way to cell size fluctuations less than this amount.

A feature of this method of volume spectroscopy is the necessity of obtaining cells in suspension in the rounded up form. In these experiments cells were treated with trypsin in order to detach the cells from the surface of the dish. Thus, although the condition of the cells at assay is constant this condition may not be the same as undisturbed cells growing on the dish. In this respect cells growing in monolayer are somewhat less suitable for such experiments than suspension cultures if cells in the latter can be kept single.

RESULTS: COMPARISON OF EXPERIMENT AND THEORY

The results of an experiment performed throughout two generation cycles of the V79-S171 subline are shown in Figure 5, and for one generation cycle of the V79-325 line in Figure 6. These figures show the development of the peak volume as a function of position in the cell cycle. Points in Figure 4 where three separate modal volumes occur at the same time were obtained by separating the volume spectra into its parent and daughter cell components as discussed earlier. The volume scale is shown in units of $V/V_0$ where $V_0$ is the mean size of daughter cells. The conversion of the volume scale from channel number to $V/V_0$ was performed by requiring the experimental data to be symmetric about $1.47 V_0 (1.50 + 1.44) V_0/2$ in the first cycle. The
experimental curves can then be extrapolated to 1.0 \( V_0 \) and 2.0 \( V_0 \) at the beginning and end of the cell cycle. Several additional experiments were performed with each cell line with results very similar to those shown in Figures 4 and 5. In both cell lines the mode of increase of cell volume with time is relatively simple and no obvious discontinuous changes in the rate of volume increase were observed. However, the degree to which this statement is true depends upon the form of the growth law ultimately established. Evidently for linear growth \( \frac{dV}{dt} \) is constant whereas for exponential growth, \( \frac{dV}{dt} \) is proportional to \( V \). Thus, considering the \( G_1 \) to S transition in Figure 6, the data could be simulated by two different linear rates of growth differing by as much as 70%, one before the transition and one after. However, if an exponential law is assumed, only a much smaller change (of the order of 20%) can be tolerated by the data. In both cases the limited length of \( G_1 \) in this cell line and the effects of "rounding" due to late dividing cells, influences this estimate. Within these limitations, changes in the pattern of macromolecular synthesis as cells progress from \( G_1 \), to S, or S to \( G_2 \) did not appear to cause changes in rate of cell volume increase. Such changes (had they occurred) were expected to be observed more easily in the V79-325 subline than in V79-S171 because of the longer \( G_1 \) period in the former.

A Chi-Squared test of fit was performed on the data to determine the degree of correlation with either the linear or exponential models. In the first cycle of Figure 5, although both linear and exponential hypotheses are accepted with a high
degree of confidence, exponential growth is slightly favored. The second generation cycle of Figure 5 appears to have a linear region. The early part of the cycle shows very clearly the rounding of the growth curve that occurs due to the spread of generation times. The Chi-Squared test, however, accepts both linear and exponential hypotheses. The growth curve of the longer generation time cell line, V79-325, in Figure 6 appears visually to match exponential growth. With the stated accuracy of $\pm 1$ channel number in peak volume resolution, the test of fit still accepts both hypotheses with greater than 95% certainty. Only when the degree of accuracy is fictitiously increased to $\pm 0.2$ channel does the Chi-Squared criterion allow for the linear hypothesis to be rejected and exponential volume increase to be accepted, both with greater than 95% confidence. This is taken to favor the exponential hypothesis in this cell line but not to prove it.

In the above comparison between theory and experiment it has been assumed that the population of synchronous cells starts with initial volume $V_0$. If mitotic cells divide over a period of time, this will have the effect of an initial lag in the volume vs. time curve. Such a lag would further reduce the opportunity to distinguish between different modes of growth in the subsequent portion of the cycle. There may be evidence of such an initial lag in the experimental data of Figure 5, but it is less apparent in the data of Figure 6.

Figure 7 shows a plot of the relative volume dispersion (i.e. relative distribution halfwidth at halfheight, $= \frac{\Delta V}{V}$) as a
function of cell cycle for the experiments shown in Figures 5 and 6. The parameter $\frac{\Delta W}{V}$ (which is about 0.83 of the coefficient of variation) remains nearly constant throughout the cycle. Anderson et al. (20) have pointed out that this is the expected behavior of a population undergoing exponential volume increase. A variety of other growth laws (but not a linear growth law) also lead to dispersionless growth. However, the experimental error associated with a determination of distribution halfwidth is greater than the 1-2% accuracy achieved in determining the peak volume of the distribution.

DISCUSSION AND CONCLUSIONS

In the two sublines of Chinese hamster lung cells tested, no relationship was detected between volume increase and changes in patterns of macromolecular synthesis within the limits of accuracy attained. In addition to the discontinuities with regard to DNA synthesis occurring at the beginning and the end of the S period, there is also the fact that in these cells RNA and protein synthesis appear to peak out about the middle to latter part of the S period and to decline thereafter (13). [This conclusion is based on the incorporation into synchronous cells of labeled uridine and labeled leucine, respectively. Since pool sizes were not specifically measured this is uncertain.] For this reason it would be of interest to undertake a volume increase vs. time experiment in synchronous cells of different lines such as HeLa cells or L cells known to have $G_1$ periods even longer than for V79-325 and with different patterns of RNA and protein synthesis (12, 16,
In both sublines of Chinese hamster cells the distinction between linear and exponential modes of volume increase is not definite. However, in the case of the long G\textsubscript{1} cell line V79-325, the statistical tests applied appear to favor exponential rather than linear growth. In addition, exponential growth is consistent with the lack of volume dispersion observed in both cell lines, whereas linear growth is not (20). Further distinctions between modes of growth would appear to depend more upon the availability of appropriate experimental material rather than improvements in the accuracy with which volume distributions can be determined by electronic apparatus.

The mathematical models discussed here indicate that the existence of a distribution of generation times in the population is responsible for the "rounding" of the curve of cell volume vs. time whatever the mode of growth. The uncertainty in the exact form of the distribution of generation times in the population, and the experimental difficulty of starting with a population of cells all of exactly the same size also make it very difficult to distinguish between different modes of growth by the direct observation of average volume with time. Koch (18) and Anderson et al. (20) have pointed this out. However, it is clear the direction experiments must take in order to yield optimum data on average volume as a function of time. In the case of mammalian cells the experimental conditions would be greatly improved if the initial distribution of synchronized cells were only a fraction of the initial population distribution used here, e.g.
the largest or the smallest subgroup. Selection of appropriate groups of cells may be feasible by electronic methods (30) or by other physical techniques, such as "STAFLO" sedimentation or electrophoresis (31).

A requirement for a suitable method of synchrony is that it should not perturb the normal mode of growth of the cell population. In the case of the mitotic selection synchrony used here there is reasonable assurance that this is the case (e.g., 32). In some other methods, biochemical inhibition of DNA synthesis for example, this may not be so (e.g. 33) and indeed, since cells continue to grow during the inhibitory period, normal growth is not to be expected immediately thereafter.

In certain non-mammalian systems further choices are possible, for example a longer period of relative growth could be observed in a system such as Chlorella where the material not only doubles but quadruples before fission. Alternatively, the effect of a distribution of generation times could be reduced or eliminated in situations where synchrony is controlled for long periods, for example in naturally synchronized sea urchin eggs or in continuously synchronized cultures of Tetrahymena (34).
II. SUBCELLULAR VOLUME KINETICS

INTRODUCTION

The mammalian cell division cycle encompasses a complicated series of events involving the maintenance of cell metabolism and the orderly increase in all subcellular components necessary for a parent cell to divide into two daughter cells. Techniques of synchronizing cultures of cells, then observing these growth events as a function of time, have provided a powerful approach to elucidating the steps in synthesis and subcellular organization necessary for cell division.

In the experiments with synchronized Chinese hamster cells described in Chapter I, smooth doubling (with time) of the average cell volume from daughter cell to next parent cell was noted (35). This chapter is concerned with questions surrounding the biogenesis of mitochondria for these same cells. Specifically, is mitochondrial replication closely linked to steps in the cell cycle or are mitochondria autonomously replicating organelles? How do mitochondrial numbers and volume change during the cell division cycle, and do such changes correlate with changes in mitochondrial activity, such as those reported by Cottrell and Avers (36) and Bosman (37)?

This present study presents experimental findings on the growth dynamics of mitochondria during the cell cycle of Chinese hamster cells. Based primarily on electron microscopic techniques, it clarifies how the increase in total cell volume is apportioned between the subcellular compartments of nucleus,
mitochondria and surrounding cytoplasm and hence provides at least partial answers to some of these questions.

MATERIALS AND METHODS

Cell culture

The cells used were the V79-S171 line of Chinese hamster cells with a generation time of 9.5 hours, subdivided into a pre-DNA synthetic period G\(_1\) of 1.5 h, a DNA synthetic period, S, of 6 h, a post-DNA synthetic period, G\(_2\), of 1.5 h, and mitosis lasting 0.5-1 h. Cells were grown in monolayer cultures attached to the surface of plastic dishes at 37°C in Minimal Essential Medium, Earle's (Microbiological Associates, Berkeley, Calif.) supplemented with 15% v/v fetal calf serum (Gibco, Berkeley, Calif.) in a humid atmosphere of 2% CO\(_2\) and air. Synchronous cells were obtained from asynchronous log phase cultures by selection for loosely attached dividing cells using a standard shake-off procedure (25, 26). Aliquots of the resulting suspension of synchronous cells were inoculated into a series of dishes and incubated at 37°C.

Cell volume and DNA synthesis

At five intervals throughout the next 10 hours aliquots were removed and labeled with a 15-minute pulse of tritiated thymidine (0.1 μCi/ml; 14 Ci/mM) followed by an autoradiographic procedure to determine the number of cells engaged in DNA synthetic activity (24, 38). Figure 8 shows a photograph taken at high magnification under the light microscope of a field of cells
from an autoradiograph prepared directly on the culture dish. Most of the cells here show superimposed grains of emulsion and hence are labeled. Several unlabeled mitotic figures are also evident including a centrally located prophase cell and an off-center telophase cell showing a chromosome bridge. Other aliquots were removed at the same times and the cells were detached from the petri dish surface by treatment with trypsin (0.03% v/v Gibco, Berkeley, Calif.). These cell suspensions were sized electronically and then fixed and prepared for electron microscopy. The sizing of cell suspensions was done using a cell volume spectrometer consisting of a 100 µ diameter aperture (Particle Data, Franklin Park, Illinois) with amplification and accessory electronics of our laboratory design, and pulse height analysis performed by a PDP-8/I computer on-line (Digital Equipment Co., Maynard, Mass.). The percentage of cells synthesizing DNA, and average cell volume, were monitored during the cell cycle as a check on the quality of synchrony and on the timing of the cell cycle.

Electron microscopy

Cells were initially fixed for approximately 24 hours in a 1% w/v solution of osmium tetroxide in 0.1 M sodium cacodylate buffer, adjusted to pH 7.4 at 4°C on a model G pH meter (Beckman Instruments, Palo Alto, Calif.). The fixed cells were then dehydrated through sequential alcohols to absolute ethanol, moved to propylene oxide and then embedded in Epon 812 using DMP 30 catalyst. Sections of 600-800 Å thickness were cut on a MT-2
microtome (Sorvall Co., Norwalk, Conn.). The sections were picked up on 200 mesh copper grids and stained with a saturated aqueous solution of uranyl acetate (6% w/v) for 30 minutes, then with Reynold's lead citrate solution for 15 minutes. Electron micrographs were made of the sections at a final magnification of 11,500X (Model HU-ll electron microscope, Hitachi Instruments, Kyoto, Japan). Figure 9 is an electron micrograph prepared in this fashion showing a central cross section through a single Chinese hamster V79-S171 cell (printed here at a final magnification of 18,500X).

Analysis of electron micrographs

The quantitative determination of cellular and subcellular dynamics from electron micrographs of populations of cells requires careful attention to technique plus the validity of certain assumptions. On each electron micrograph the whole cell cross sectional area was partitioned into nuclear, mitochondrial and cytoplasmic components on the basis of the distinguishable morphology of each of these components. This was done for all sections of cells appearing on the photograph, including portions of cells cut off by the edge of the photograph, and sections which did not include any portion of the nucleus. The cross sectional area of each component was then determined using a polar planimeter by tracing separately the perimeter of the whole cell and the perimeter of the nucleus. The area of each mitochondrion was determined by measuring the major and minor axes and computing the area of the equivalent ellipse. The number of mitochondria
within each measured area of cytoplasm was also noted. Mitochondrial sections larger than 2 mm in diameter (on the photograph) were recorded; smaller sections than these could not be reliably recognized as mitochondria.

At each experimental point approximately fifteen 8" x 10" photographs representing 6000 μ² of cell area were studied. Each photograph was taken of a random field chosen blindly so as to avoid arbitrarily selecting 'eye-catching' fields. (For example, the eye would be automatically drawn to fields with a large nucleus, since on the electron microscope viewing screen this forms a marked area of contrast. Preferential selection of such fields would clearly lead to a bias in the ratio of observed nuclear to cytoplasmic area.) Care was also taken to select sections from the embedding pellet so that no two sections were cut within 20 μ (about two cell diameters) of each other, thus insuring that each section was cut through a different group of cells.

RESULTS

The results of the progression with time of the percentage of cells labeled with tritiated thymidine as determined by (i) autoradiography (percent labeled index) and (ii) the average cell volume are shown in Figures 10a and 10b, respectively. The solid lines through the data points are the predictions of a computer model of the synchronized population based on the distribution of cell generation times (35). These quantities were monitored as a check on the quality of synchrony and on the timing of the cell
cycle.

A high yield of cells from the synchrony procedure was necessary to produce the $5 \times 10^6$ cells required for this experiment, which led to a corresponding sacrifice in the best quality of synchrony normally attained by the mitotic selection procedure. The synchrony as measured by the labeling index (Figure 10a) showed a rise from only 30 to 68%. The same experiment showed considerably better synchrony in terms of cell volume changes (Figure 10b), with the data fit by a mathematical mode assuming a 15% coefficient of variation in the distribution of cell generation times. This is in comparison with the best results from Chapter I (Figure 5), which showed a change in the labeling index of 80% with a coefficient of variation of 9.5%. In addition, the G₁ period of the cell cycle was found to be 3 hours long rather than the normal 1.5 hours characteristic of this cell line. Such a delay in G₁ has been noted on several occasions following a large shake-off procedure as a consequence of longer times required in handling large cell batches. Following this induced delay, the events in the cell cycle proceeded with normal timing.

The results of measurements of the individual subcellular component areas are expressed as the nuclear area fraction, $N/(N+M+C)$, and the mitochondrial area fraction, $M/(N+M+C)$, where N, M and C are the nuclear, mitochondrial and cytoplasmic areas respectively. The mitochondrial number density was also measured and is denoted as $M_n/(N+M+C)$, where $M_n$ is the number of mitochondria observed within the whole cell area $(N+M+C)$. The uncertainty in the mean value obtained for each of these ratios is
derived from the Poisson statistical uncertainty of counting individual units of area. Specifically the error in the ratio \( X/Y \) is given by:

\[
\Delta X/Y = \pm \frac{X + \sqrt{X}}{Y + \sqrt{Y}}
\]

Fifteen photographs sampling 6000 \( \mu^2 \) of cell area was sufficient to achieve a statistical uncertainty of less than 10% of the value of the mean.

The measurements of the individual subcellular component areas were derived from examining approximately 6000 mitochondria in 500 cells (or portions of cells) in 100 photographs. The results are shown in Figures 11a, 11b and 11c as a function of time during the cell cycle. The timing of the phases of the cell cycle as determined from the results of Figure 10, is also shown.

The partitioning of the increase in cell volume during the cell cycle into subcellular compartments of nucleus, mitochondria and cytoplasm occurs in such a way that the nuclear area fraction, \( N/(N+M+C) \) remains constant, i.e. the nucleus changes in size at the same pace as the whole cell. However the mitochondrial area fraction, \( M/(N+M+C) \), shows significant decreases in \( G_1 \) and \( G_2 \) compared to its value in mid \( S \) (Fig. 11b), and a parallel change is also seen in the independent parameter, the mitochondrial number density, \( M_n/(N+M+C) \), (Fig. 11c). Chi-squared test of goodness of fit show the curves of Figures 11b and 11c to deviate significantly from (horizontal) straight lines obtained from a least squares fit, with \( p = 0.5 \) and \( p < 0.001 \), respectively. The curve for the nuclear area fraction versus time (Fig. 11a),
however, is fit by such a line, \( p > 0.95 \).

**Absolute Organelle Volumes--Synchronous**

A more conventional and quantitatively useful form of the data can be obtained by converting the nuclear and mitochondrial area fractions into their respectively absolute volumes. The actual volumes of the nucleus and mitochondria are calculated from the respective area fractions plus a knowledge of the average whole cell volumes as determined by the electronic sizing method. The area fractions represent the average nuclear and mitochondrial areas per element of whole cell cross sectional area in randomly oriented sectional planes through the cells. Calculation of the nuclear volume is performed by referencing the cell with respect to a z axis perpendicular to the nuclear areas in the serial cross sections and integrating over this area. That is

\[
\mathcal{N} = \int \frac{N}{N+M+C} A(z) \, dz
\]  

(2)

where \( \mathcal{N} \) is the volume of the nucleus; \( A(z) \) is the cross sectional area of the cell as a function of distance along the z axis; and \( \frac{N}{N+M+C} \) is the nuclear area fraction in that cross section. In order to use eq. (2) for the data of this chapter, replace the function, nuclear area fraction, by our measured mean value obtained for the nuclear area fraction from many randomly oriented planes. Thus, eq. (2) becomes:

\[
\tilde{\mathcal{N}} = \frac{N}{N+M+C} \int A(z) \, dz
\]  

(3)

where \( \tilde{\mathcal{N}} \) is the mean nuclear volume.
The integral portion of eq. (3) is simply the mean whole cell volume, $V$. Thus eq. (3) is equivalent to:

$$\tilde{n} = \left[\frac{N}{N+M+C}\right] \tilde{V} \tag{4}$$

Similarly for the mean total mitochondrial volume within a cell:

$$\tilde{m} = \left[\frac{M}{N+M+C}\right] \tilde{V} \tag{5}$$

The absolute mean whole cell volume $\tilde{V}$, in cubic microns is calculated from the electronically measured relative volumes, $V/V_o$ (see Fig. 1b) by the expression:

$$\tilde{V} = (710 \mu^3/1.44 \text{ relative units}) V/V_o \tag{6}$$

The multiplicative factor in eq. (6) is determined from calibration of the electronic volume sizing apparatus. This calibration factor is obtained by matching mean experimental volume ($710 \mu^3$) with the theoretical mean cell volume of $V/V_o = 1.44$ for a population with an exponential mode of volume increase (see reference (35)).

It is also possible to estimate the total number of mitochondria per cell from the number density per cross sectional area. The volume number density is simply the area density raised to the $3/2$ power hence:

$$m_n = \left[\frac{M_n}{N+M+C}\right]^{3/2} \tilde{V} \tag{7}$$

These formulas for absolute volumes and number do not include corrections for two second order effects. Firstly, these derivations assume infinitely thin sections, whereas actual sections
were between 600 and 800Å thick. Thus, mitochondria within a given cross sectional cut may not all be within the same mathematical plane, leading to an overestimation of total mitochondrial volume by eq. (5) and also of the mitochondrial number by eq. (7). The size of this second order effect can be estimated as follows: The number density of mitochondria per cell area, averaged for all experimental data is $0.25/\mu^2$. This implies an average nearest neighbor distance between mitochondria of $(1/0.25)^{1/2}$ or $2\mu$. Since the sectional thickness is only $1/3$ this average distance, the 'overcounting' will be small. A second factor affecting the accuracy of eq. (7) but in the opposite direction, is the fact that mitochondrial sections smaller than 2 mm in diameter on electron micrographs were not counted because they have too indistinct a morphology to be scored as mitochondria. For an average mitochondria represented by a 1 μ diameter sphere, this correction is less than 2%.

The results of these calculations from the organelle area fractions are given in the form: mean nuclear volume, $\bar{m}$; mean total mitochondrial volume per cell, $\bar{v}$; and average number of mitochondria per cell, $\bar{m}_n$. These parameters are shown in Figures 12a, 12b and 12c, respectively, as a function of time in the synchronized cell division cycle.

**Absolute Organelle Volumes--Asynchronous**

The mean values of the nuclear volume, total mitochondrial volume and number of mitochondria per cell in an asynchronous log phase population of the cells were determined in two
experiments. In the first experiment (#G-43), 17 electron micrographs of such cells were scored for nuclear and mitochondrial parameters. Organelle volumes and numbers were calculated directly from these data and the results are given in Table 2.

A second and indirect determination of these values can be made by averaging appropriately weighted data obtained from the 75 electron micrographs of the synchronized cells (experiment #G-45).

The value of any parameter X for an asynchronous population can be calculated from the value obtained in synchronous cells by the following formula:

$$X_{\text{asyn}} = \lim_{N \to \infty} \left\{ \begin{array}{c} X(T_1) \int_0^{T_1+1/2(T_2-T_1)} f(t) \, dt \\ \vdots \\ X(T_2) \int_{T_1+1/2(T_2-T_1)}^{T_2+1/2(T_3-T_2)} f(t) \, dt + \cdots \\ X(T_N) \int_{T_{N-1}+1/2(T_N-T_{N-1})}^{T_N} f(t) \, dt \end{array} \right\}$$  \hspace{1cm} (8)

where \(X(T_1), X(T_2), \ldots, X(T_N)\) are the N determinations of X made during the cell cycle, i.e. between the times \(t=0\) and \(t=T_0\), the generation time. The weighting function \(f(t)\) is the normalized age distribution of a log phase population:

$$f(t) = \frac{2\ln 2}{T_0} \exp \left( -\ln 2(t/T_0) \right)$$  \hspace{1cm} (9)

The integral of \(f(T)\) over the single time period
\[ T_{i-1} + \frac{1}{2} (T_i - T_{i-1}) \text{ to } T_i + \frac{1}{2} (T_{i+1} - T_i) \] is the proportion of the log phase population having cell ages in that interval. Multiplying this weighting factor by the value \( X(T_i) \) measured in the middle of this interval, and summing over all measurements during the cell cycle, gives an estimation of the parameter \( X_{\text{asyn}} \), characteristic of the asynchronous population from which the synchronized cells were selected. The estimate of \( X_{\text{asyn}} \) improves with the number of points taken, i.e. with \( N \).

The values for the nuclear volume and mitochondrial volume and numbers obtained by this indirect method from the data of Figure 12 are likewise given in Table 2.

The uncertainty in each of the values given in Table 2 is estimated by a propagation of errors formula that includes the 3% imprecision in the calibration of the electronic sizing procedure, the 10% error in determining relative cell volume, \( V/V_o \), and the 10% error in determining the organelle area fractions. These later two errors are strictly the uncertainties due to Poisson counting statistics (see eq. (1)). These units thus represent the uncertainties in determining the mean values of these parameters rather than the range of variation in the cell population.

The only significant differences between the two sets of values given in Table 2 are those seen in the number of mitochondria per cell and in the derived parameter—the mean volume per mitochondrion. This difference in the number but not in the
total volume of mitochondria per cell points up the need for additional investigation of mitochondrial dynamics in cell culture over longer time spans than one generation.

DISCUSSION

The evidence obtained in this study suggests that while the nucleus increases in size in pace with whole cell growth, the mitochondria exhibit a more complex kinetics. The observed changes in mitochondria represent an appearance and disappearance of structures recognizable as morphologically intact mitochondria. In eucaryotic cells mitochondria have been thought to have originated as parasites as evidenced by their many similarities to bacteria (39), though in yeast, they have been shown to be dependent on nuclear genes (40). These ideas suggest a simple model of mitochondrial replication by binary division, either synchronously or asynchronously, leading to a doubling within one generation time. The present findings of a decreased mitochondrial volume and number in G₁ and especially G₂ when the cell is approaching its maximum volume prior to division are not consistent with such a model.

In yeast it is known that mitochondria can undergo a reversible reduction to promitochondria when exposed to anaerobic conditions (41). Such promitochondria are normally not recognizable under the electron microscope. The decreases in mitochondria which we observed could be due to a similar mechanism whereby mitochondria not in an active state are not morphologically recognizable. Other hypotheses of mitochondrial dynamics, such
as disassembly and assembly by the cell itself (rather than autonomous division) are also consistent with the data.

Studies on mitochondrial dynamics in synchronized microbial cultures demonstrate that no single hypothesis of mitochondrial biogenesis is consistent with all the observed patterns. Osumi and Sando (42) report, from a light and electron microscopic study of synchronized cultures of *Schizosaccharomyces pombe*, that nuclear division begins at 1/5 and ends at 4/5 of the cell cycle, while mitochondrial division begins at 2/5 and ends at 3/5 of the cycle. Cottrell and Avers (36) find a different pattern in synchronized *Saccharomyces cerevisiae*, which they cannot account for in terms of such a model of synchronized mitochondrial division. They find a linear increase during the cell cycle in numbers of mitochondria per electron microscopic field, which they interpret as a constant nonsynchronous biogenesis of mitochondria. In *Neurospora*, Hawley and Wagner (43) report increases in numbers of mitochondria synchronous with mitotic activity.

Studies in mammalian cells on the variation in actual numbers or volumes of mitochondria are sparse despite the early work of Allard et al. (44). Using a light microscopic technique these authors observed an increase with time in numbers of mitochondria in regenerating rat liver cells. More recently in a quantitative study of electron micrographs of normal human liver biopsy material Tauchi and Sato (45) describe a decrease in number but an increase in size of mitochondria with advancing age.

Biochemical events associated with mitochondrial biogenesis during the cell division cycle have been more completely investigated.
Thus, for Chang liver cells synchronized by cold shock, the rate of synthesis of mitochondrial DNA has been observed to be maximal in G₂ (46). For mouse L5178Y cells synchronized by excess thymidine and colcemid, Bosman (37) found the rate of mitochondrial DNA synthesis to be greatest in S and G₂. Rabinowitz (47) has reviewed many of the experiments on mitochondrial nucleic acids and discussed their relation to the problem of mitochondrial biogenesis. However, an important variable in these studies is the unknown effects of the mitochondrial population of the physical and chemical methods of synchrony used. I felt it desirable to use a selection procedure for synchronization in order to minimize introduction of an unknown perturbation in the mitochondrial dynamics.

The significant changes in mitochondrial dynamics were clearly seen despite the less than optimal degree of synchrony of the cells (resulting from the large scale preparative requirements). Technical improvements and extension of those studies to other cell culture systems will surely be worthwhile. Further studies are also clearly indicated to relate mitochondrial dynamics as observed here, to mitochondrial metabolism during the cell cycle. Finally, there is a pressing need to clarify the physical mode of replication of mitochondria in order to elucidate a more complete picture of the relationship between the whole cell and its organelles.
III. STATIONARY PHASE CELL CYCLE DISTRIBUTION

INTRODUCTION

Cultures of mammalian cells from established tissue culture lines are widely useful in the investigation of various properties of mammalian cells. The procedures employed in maintaining an established cell line in culture are designed to propagate the cells at a maximal rate by supplying the culture with an ideal environment of nutrients, gas tensions, humidity and temperature. In these conditions cell number increases exponentially with time, doubling about every ten hours for a typical mammalian cell line such as Chinese hamster lung cells of V79 origin (48). The doubling time in cultures with no cell loss approaches the cell generation time (49). The cell generation cycle defines a sequence of events which occur between the time a fissioning cell separates into two daughters until the time that one of the daughters then fissions into two cells.

The purpose of this third chapter is to describe, in terms of the cell generation cycle, the sequence of events that occurs, first when cells in culture enter stationary phase and are maintained there for some time, second when renewed growth conditions enable a return to log phase, and third the dependence of these sequences upon the type of growth medium.

Nutritive Capacity and the Onset of Stationary Phase

The culture of mammalian cells from an established cell-line in a fixed amount of nutrient medium is characterized by a pattern
of population growth separated into three phases: lag, logarithmic and stationary. Stationary phase describes the condition of a culture in which log phase growth has ceased, i.e. cell number no longer increases exponentially. The cessation of an exponential increase in cell number is usually due to a depletion of the nutrient supply or less commonly to density dependent inhibition. Normally when stationary phase cultures are diluted and transferred into fresh medium, growth of the population resumes, and exponential increase in cell number reoccurs. The duration of each phase and the doubling time within log phase are a property of the nutrient medium and culture conditions. A useful parameter can be defined which characterizes the quantitative capability of the cell medium to support exponential increase in cell number during log phase. This parameter is called the nutritive capacity, $N_c$, and is obtained by integrating the experimentally determined growth curve, cell number versus time, $N(t)$, from the start, $T_1$, to the end, $T_f$, of the log phase of growth. The value of the integral:

$$N_c = \frac{1}{v} \int_{T_1}^{T_f} N(t) dt$$

is a measure of the maximum number of cell-hours of log phase growth that can be supported by the given volume, $V$, of medium used in the experiment. The transition point, $N(T_f) = N_f$, from log to stationary phase is located by the intersection of the
straight line exponential portion of the growth curve (in a semi-log plot) with a horizontal tangent to the growth curve at the point of maximum cell number. This point was originally proposed as the boundary between log and stationary phase by Watanabe and Okada (50). The transition point from lag to log phase is found similarly.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

The cells used were the V79-S171 subline of Chinese hamster cells originally grown from female lung tissue (24). The generation time of these cells is approximately 9.5 to 10.0 hours. The mitotic cycle is subdivided into a pre-DNA synthetic period, \( G_1 \), of \(~1.5\) to \(2.0\) hr.; a DNA synthetic period, \( S \), of \(~6\) hr.; a post-DNA synthetic period, \( G_2 \) of \(~1.5\) hr.; and a 0.5-1.0 hr. mitotic period, as reported earlier (35). Cells were grown in monolayer cultures attached to the surface of plastic dishes (60 mm diameter) at \(37^\circ\)C in a water saturated atmosphere of 2% \(CO_2\) and air. The nutrient medium used was one of two very similar media, denoted HUT-15 or EM-15 (51) both containing 15% fetal calf serum. Table 1 lists the composition of EM-15 medium, a minimal medium, as prepared at Argonne National Laboratory by W. K. Sinclair. The composition of EM-15 medium is minimal in the sense that no one ingredient can be completely omitted without a significant worsening of the growth properties, however the quantitative amounts of each ingredient are not the minimal amounts necessary
for growth. The HUT-15 medium differs from EM-15 medium by a 2.5% v/v enrichment of NCTC 109 nucleotide, vitamin and amino acid mixture (Difco Laboratories, Detroit, Michigan) and 2% v/v of 0.03% trypsin (Grand Island Biologicals, Grand Island, Michigan).

**Measurement of Dynamic Population Parameters**

Cell concentration and average cell volume were monitored by means of a Coulter aperture and a 400 channel analyzer described previously (35). The rate at which cells in stationary phase re-enter the cell cycle when provided with fresh medium was determined by noting the rate of appearance of cells at mitosis. Two methods were employed to measure the flux of cells at mitosis. In one, cultures supplied with fresh medium and the mitotic inhibitor Colcemid (Ciba Pharmaceutical Company, Summit, New Jersey) at a final concentration of 0.2 µg/ml were used to measure the cumulative mitotic index. In theory, changes in the cumulative mitotic index with time represent the net flux of cells into mitosis; however in practice Colcemid does not arrest 100% of all cells arriving at metaphase as recognizable metaphase figures. In log phase cultures the cumulative mitotic index fails to show the actual flux of cells into mitosis after only five hours. In these experiments of cells resuming growth from stationary phase, the length of time for which Colcemid maintained a nearly 100% block was significantly greater than for log phase cells as revealed by other methods of inferring the flux of cells reaching mitosis. Nevertheless because of the unknown rate of loss of recognizable metaphase cells with time under these experimental
conditions the cumulative mitotic index is possibly an incomplete measure of the cells at metaphase. The second method of measuring the flux of cells at metaphase was to score for mitotic cells in cultures free of any mitotic inhibitor. This is an "instantaneous" measure of the flux of cells in mitosis and when adjusted for the finite duration of mitosis the mitotic index represents the derivative of the cumulative mitotic index versus time curve. An advantage of the cumulative mitotic index over the instantaneous mitotic index in these experiments is due to the fact that the first generation of cell growth after stationary phase is long (~24 hours). Cells that resume growth rapidly if not blocked at mitosis will progress through the cell cycle a second time and reach mitosis again while cells that are slow to resume growth are still passing through their first mitosis. Both methods were used and equivalent results were obtained within the limits of their accuracy.

Tritiated thymidine in a concentration of 0.1 μCi/ml, 14 Ci/mM specific activity, was used in a 15 minute pulse label followed by radioautography to determine the percent of cells synthesizing DNA. Tritiated uridine (0.5 μCi/ml, 29 Ci/mM) and leucine (4.0 μCi/ml, 5 Ci/mM) were similarly used to measure the number of cells engaged in RNA and protein synthesis respectively. An exposure period of one week was employed in the radioautography procedure; the background was determined to average less than 4 grains/cell and the percent of cells synthesizing either DNA, RNA or protein was taken to be the percent of cells with 4 gains/cell or greater for the respective labeled precursor. This level of
exposure also permits a count of number of grains per labeled cell to be made and thus a determination of relative rates of synthesis of each of these macromolecules provided pool size remains constant. These radioautography plates when stained with iron-hematoxylin were also used to score for mitotic cells to determine the mitotic index. Figure 8 (discussed earlier) is an example of a photograph of such an autoradiographic plate.

Viability of cells was determined by scoring macroscopic colony formation from single cells about 10 days after inoculation.

RESULTS

Nutritive Capacity and the Onset of Stationary Phase

Figure 13 shows the growth curves of Chinese hamster V79-S171 cells grown in four variations of EM-15 medium in an experiment performed in 1966 by W. K. Sinclair at the Argonne National Laboratory. He employed the basic EM-15 medium, supplemented by either 2% v/v of 0.03% trypsin solution or 2.5% v/v NCTC-109 enrichment medium or both these additives. Figure 13 demonstrated the progressive increase of maximum cell number in stationary phase that results from medium enrichment. Table 3 lists the nutritive capacities that W. K. Sinclair and I now calculate for each of these media. Quantitatively equivalent results were obtained when I repeated this identical experiment at Donner Laboratory in 1970. Either trypsin or NCTC-109 in fractional quantities can substantially increase the nutritive capacity of EM-15 medium (i.e. increase the time in log phase) and when used
together their effects sum. Elkind (private communication) has previously noted this ability of trypsin to increase final cell number. The parameter \( N_c \) permits this effect to be quantititated.

Figure 14 indicates the results of an experiment on the effect of varying the percent fetal calf serum (Grand Island Biologicals) in Minimal Essential Medium (52). The nutritive capacity, \( N_c \), and the cell population doubling time, \( t_d \), are plotted as a function of percent fetal calf serum. The nutritive capacity was found to be directly proportional to the percent serum up to 20% with continuing slight increase to 35%, and then to decrease at even higher concentration. The linear portion of the curve from 0 to 20% has an intercept at the origin with a slope equivalent to the doubling of nutritive capacity for every additional 10% of serum. The population doubling time, \( t_d \), was found to have hyperbolic dependence on the percent serum, with \( t_d \) asymptotically approaching infinity for zero percent serum and approaching 8.5 hours for greater than 20% serum. Hahn and Bagshaw (53) have described the dependence of cell population doubling time, \( t_d \), on percent serum and attributed the change in \( t_d \) to be mainly due to a shortening of the \( G_1 \) period in the cell cycle.

A number of experimental determinations of the nutritive capacity of three different cell media and the means and standard deviations of \( N_c \) are given in Table 4. The variance of \( N_c \) about the mean for successive experiments is large (CV = 30%). However in multiple measurements of \( N_c \) within a single experiment, \( N_c \) is more precisely determined (CV = 10%). The nutritive capacity has
been used in our laboratory as a parameter to test the compare the growth properties of different lots of fetal calf serum and various types of cell culture media. The nutritive capacity has also been used to predict the time of onset of stationary phase for any culture where the initial cell concentration and volume of nutrient medium are known.

**Time Course From Log to Stationary Phase**

As cells progress from log to stationary phase, the modal cell volume of the population as determined by electronic sizing, decreases by about 20% for cells in HUT-15 medium. The modal cell volume for cells grown in EM-15 shows a slower rate of decline with a net decrease of about 10% as cells enter stationary phase, as is shown in Figure 15a. (In several experiments an initial brief increase in cell volume followed by a 10% decrease was noted for cells entering stationary phase in EM-15.) This behavior, which was noted during routine tissue culture procedures prior to the start of these experiments, was the motivation for using both of these cell media throughout the investigation.

The transition from log to stationary phase is also characterized by a drop in plating efficiency, mitotic index and labeling index; that is, the percent of mitotic cells and the percent of cells which take up labeled DNA precursor $^3$HdR from the medium. Figure 16 summarizes these changes. Note the differences between HUT-15 and EM-15 media, especially the persistence of cells synthesizing DNA in mid and late stationary phase in EM-15. The percent of cells synthesizing RNA also drops in proceeding
from log to stationary phase for both media, however the percent of cells synthesizing protein does not decrease from 100% in either medium upon entering stationary phase.

**Growth Resumption Experiments**

Cell populations in early, mid and late stationary phases were trypsinized and diluted in fresh medium to a concentration of $5 \times 10^4$ cells/plate. Mitotic index, label index and cumulative mitotic index in cultures blocked with Colcemid were assayed as a function of time during growth resumption. Figure 17 shows these results for a growth resumption experiment from early stationary phase. Error bars on points represent $\pm 1$ s.d. due to Poisson counting statistics. For cells in both HUT-15 and EM-15 media the cumulative mitotic index initially rises sharply followed by a period of 8 to 10 hours in which there is very little increase, followed thereafter by a second steep rise (Fig. 17b). Bursts of mitotic activity (Fig. 17c) in cultures containing no Colcemid correlate with the increases in cumulative mitotic index. The mitotic index falls to 0.5% during the plateau in cumulative mitotic index. Also during this plateau the percent of labeled cells increases markedly. This suggests that the first increase in cumulative mitotic index is due to $G_2$ cells. No increase occurs in the subsequent period because only a small number of cells are in $S$ under these conditions. The second increase in cumulative mitotic index is due to $G_1$ cells reaching the block.

The cumulative mitotic index and its derivative, the
instantaneous mitotic index, along with the simultaneously measured label index in cells resuming growth, also permits a determination of the residence time of cells in each of the cell cycle compartments in this first generation after stationary phase. The second peak in the mitotic index occurs 2 to 5 hours before the corresponding rise in cumulative mitotic index. This is likely due to the long exposure to Colcemid, which is known to have a retarding effect on the blocked cultures. Therefore the time duration of passage through the first cycle after stationary phase was determined from the mitotic index curve alone (Fig. 17c). Each of the $M_1$, $G_1$, $S$, $G_2$, portions of the cycle are approximately twice as long as in log phase. Cell number and cell volume were also measured during growth resumption and by the end of this first generation the average cell volume had returned from the characteristic stationary phase value to the log phase value (see Fig. 15b).

Results from $^3$HtdR labeling experiments in EM-15 medium showed that 40% of the population of mid stationary phase cells were synthesizing DNA (as compared to 10% or less for HUT-15). Comparison between grain counts for log and stationary phase labeled cells showed that these stationary phase cells were synthesizing DNA at only 55 ± 15% of the rate associated with log phase cells. Yet the growth resumption experiments demonstrated that these stationary phase cells in EM-15 medium which were synthesizing DNA did not later reach mitosis when provided with fresh medium. It appears therefore that the subpopulation of cells in EM-15 medium which synthesize DNA during stationary phase are
not capable of resuming cell division in fresh medium.

Figure 16 summarizes the changes that occur when cells progress from log to stationary phase for the two media: (A) HUT-15, (B) EM-15. The right hand column shows histograms representing the distribution of cells within the cell generation cycle in log phase, and early, mid and late stationary phase as determined by these growth resumption experiments. The height of the bars is proportional to the fraction of cells in each compartment of the cycle. The fraction of cells in $G_1$ and $G_2$ is determined from a graph of cumulative mitotic index versus time as in Figure 17b; the cumulative mitotic index at the first plateau is directly proportional to the fraction in $G_2$, the differences between the first and second plateaus, the fraction in $G_1$. The fraction of cells in $G_1$ and $G_2$ was also estimated by taking the relative areas under the two peaks in the instantaneous mitotic index curve versus time (Fig. 17c). Equivalent results were obtained. The fraction of cells in S is determined by the numerical average of two factors: (a) the label index of the starting stationary phase population, and (b) the rise in the percent cumulative mitotic index during the S phase of growth resumption. This procedure was used for experiments in HUT-15 medium where the two values from methods (a) and (b) agreed within limits of accuracy. For cells in stationary phase in EM-15 medium only method (b) which indicated zero percent cells in S was used, since although 40% of stationary phase cells in EM-15 medium take up $^3$HtdR label these cells are incapable of future cell division. The fraction of cells in M is determined by the mitotic index of the starting
stationary phase population. These are the procedures by which the results from data shown in Figure 17 are compiled into the histograms shown in Figure 16. The histograms represent the average of the results from several such experiments. The histogram for the distribution of log phase cells within the compartments of the cell generation cycle was determined from a knowledge of the duration of each portion of the cell cycle as measured in synchronous cultures (35). The conversion from a measurement of the durations of the cell cycle compartments in log phase to a measure of the fraction of cells in each compartment was made assuming a steady-state exponential distribution of cell ages within the cell generation cycle. Nachtwey and Cameron have written a review of these procedures from studying the kinetics of log phase cells (4).

For cells in HUT-15 medium in early stationary phase, \( G_1 \) cells represent the largest fraction, with a substantial proportion in \( S \) and \( G_2 \). For cells in EM-15 medium in early stationary phase the fraction in \( G_2 \) is greater than the fraction in \( G_1 \). Less than 1% of the population is observed in mitosis in stationary phase for cells in both media. As time in stationary phase increases, the relative number of cells in \( G_1 \) increases as compared to numbers in \( S \) or \( G_2 \). This is true for both media, but the shift to \( G_1 \) occurs more rapidly in HUT-15 than in EM-15.

The plating efficiency also drops drastically with time in stationary phase. By the time the population is predominantly in the \( G_1 \) portion of the cycle (late stationary phase) the plating efficiency drops to 0 to 5%. That is, only a few cells have the
ability to resume unlimited division when transferred to fresh medium. However plating efficiency results must be interpreted differently for a non-proliferating cell population. Plating efficiency in stationary phase measures the percent of cells which will return to continuous unlimited division and give rise to a clone when supplied with fresh medium at a low cell density. In late stationary phase although only a few percent of cells can revert to clonogenic growth, a sizeable fraction of the population is capable of some growth and at least one cell division. This is demonstrated by the observation that the cumulative mitotic index of cells resuming growth from stationary phase rises to 35%, indicating that roughly one-third of the population is capable of at least one division, though few will give rise to clones.

DISCUSSION

The occurrence of stationary phase in Chinese hamster V79-S171 cell cultures in two related media, HUT-15 and EM-15, has been described by means of a parameter called the nutritive capacity, $N_c$, which measures the depletion of the nutrient medium. The numerical value of $N_c$ is the number of cell-hours per ml. of medium for which log phase growth can be maintained as defined in equation (1). Table 4 shows that $N_c$ is approximately 30% greater for HUT-15 medium than for EM-15.

The nutritive capacity may be employed to determine whether the growth of a cell culture is limited by cell medium nutrients or other factor(s). Many reports have claimed that repeated
feeding of cells does not stop a culture from entering stationary phase and the conclusion is put forward that, in these experiments, stationary phase is due to cell crowding or cell density dependent inhibition alone and not to nutrient deficiencies. This conclusion may be false if feeding were inadequate and this is often, if not usually the case. Using the criterion of nutritive capacity a culture containing $10^7$ cells in 5 ml. of MEM medium will exhaust that amount of medium in approximately 4 hours. To test this hypothesis, an experiment was performed in which log phase cells were kept in log phase by changing the cell medium as often as dictated by the nutritive capacity. Thus when $10^8$ cells are present for example, 5 ml. would last only 0.6 hours. It was possible to maintain a culture in log phase with only a slight decrease in population doubling time to a cell density ten times higher than the normal for an unfed experiment. At this point even though the beginning of stationary phase was not indicated, the experiment was stopped because only continuous flushing of the culture with fresh medium would satisfy the criterion for avoiding the depletion of nutrient medium. That is, nutrient deficiency is the cause of the onset of stationary phase even in apparently fed cultures. The value of $N_c$ in cell-hours per ml. of medium is proposed as a convenient test for determining whether a cell culture is limited by cell medium nutrients.

A series of events occurs when Chinese hamster V79-S171 cells reach stationary phase due to nutrient medium depletion that can be described in terms of the cell generation cycle. These events differ for minimal (EM-15) as compared with enriched (HUT-15)
medium. As a culture progresses from log to early, mid and eventually late stationary phase the relative proportion of cells in the $G_1$ compartment increases steadily for cells grown in HUT-15 medium (Fig. 16a). For cells grown in EM-15 medium (Fig. 16b), there is an initially larger component of cells in $G_2$ than in $G_1$ for early stationary phase. Cells grown in EM-15 medium also ultimately end up mainly in $G_1$ but the sequence of stages differs.

This overall pattern of redistribution in the cell cycle during stationary phase has been observed by other investigators, but the exact quantitative nature of the redistribution has varied in nearly every case. Watanabe and Okada (50) report that stationary phase in a suspension cell culture of the mouse lymphoma line L5178Y cells was due mainly to a prolongation of the $G_2$ portion of the cycle. Chapman, Todd and Sturrock (54), working with the V79-379a line of Chinese hamster cells, reported finding 90% of a stationary phase culture in $G_1$ only. Tobey and Ley (55) using Chinese hamster cells (CHO line), also found a 90% accumulation in $G_1$ for a stationary phase population. They also investigated this question with the use of a high-speed flow microfluorometric technique and reported that these cells possessed the total DNA content expected of log phase $G_1$ cells. Hahn, Stewart, Yang and Parker (56) using HA2 Chinese hamster cells reported stationary phase populations mostly in a $G_1$ or $G_1$-like compartment of the growth cycle. They also observed a portion of the stationary phase population in a DNA synthetic compartment of much longer than normal duration. Webster and Van't Hof (57) studying plant tissue (pea root meristems) induced stationary phase by
carbohydrate starvation with a resulting accumulation of cells in $G_1$ and $G_2$. Van't Hof (58) has also shown that feeding bursts can cause a stationary phase population of these cells to enter and then to be arrested and maintained in the DNA synthetic portion of the cycle. A review of the pre-1968 literature on this topic is given by Epifanova and Terskikh (59). The brief review given here is mainly to demonstrate the apparent variability that can occur in the redistribution of cell populations within the cell cycle compartments during the onset of stationary phase. Especially to be noted is that in early stationary phase in less rich medium more cells may reside in $G_2$ than in $G_1$. Thus the type of medium and the conditions employed, especially the length of time in stationary phase, may strongly influence the distribution of the population.

The kinetics of growth resumption from stationary phase when cells are diluted into fresh medium is also of interest for the study of cell cycle control since to a degree this growth resumption is synchronized. Tobey and Ley (55) and also Chapman, Todd and Sturrock (54) have suggested this as a means of producing synchronized populations. In the present experiments a degree of synchrony was also found, as measured by: (i) the rate of arrival of cells at a metaphase block from cultures resuming growth, and (ii) labeling patterns (see Fig. 17). The Blumenthal-Zaler index of synchrony (27), obtained from a plot of cell number versus time for the experiment shown in Figure 17 was only $35 \pm 10\%$ however. This is low compared with results from mitotic selection (26). This synchronized progression of cells in a
growth resumption experiment was not associated with a synchronized pattern of increase of average cell volume as reported in Chapter I for cultures synchronized by mitotic selection. Thus there can be an uncoupling of progression through the cell cycle in terms of metabolic changes, and progression in terms of increase in average cell volume. This point was also discussed by Tobey and Ley (55) and has been studied in a specific experiment by Fox and Pardee (60).

Even though the first generation cycle in cells resuming growth from stationary phase is to a degree synchronized, this first cycle is approximately 24 hours long instead of the 10 hour generation time associated with log phase cells and can be expected to be irregular in other ways. Furthermore, the population of cycling cells in a growth resumption experiment includes a predominantly large subpopulation which has only a limited future of cell division as revealed by plating efficiency tests. Thus growth resumption from stationary phase would not appear to be a suitable source of synchronized cells. The growth resumption of mammalian cells from stationary phase is, however, representative of the recovery of cells from stress, in this case that resulting from starvation.

The description of cells entering stationary phase, and of subsequent growth resumption, can be accomplished in terms of a dynamic redistribution within the metabolic compartments of the cell generation cycle indicating the usefulness of this type of analysis for both proliferating and non-proliferating cell populations resulting from a variety of experimental conditions.
GENERAL DISCUSSION

As mentioned in the introduction, to the biophysicist the problem of cell growth and division 'begs for expression in terms of quantitative measures of rates of processes and their interactions in time.' The experiments described and discussed in chapters I, II and III have attempted to do this. Other authors have formulated descriptions of cell growth and division in the same spirit, based on their experiments and interpretations. These quantitative studies form a valuable literature in the field of cellular biophysics.

A group at the Los Alamos National Laboratory, Anderson, Bell, Petersen and Tobey, have in a series of six articles (7, 8, 19, 20, 61, 62) developed an involved mathematical model of cell growth and division with some correlated experimental data. They maintain that cell volume in Chinese hamster cells increases exponentially with cell density remaining constant during the division cycle. The experimental work presented by this group is impressive for its accuracy and the degree of synchrony which they were able to obtain by a massive multiple shake-off procedure. Their understanding and discussion of the pitfalls and problems in electronic cell volume spectroscopy is also very useful to workers in this field. The mathematical model of a synchronized cell population which they develop from quite general assumptions is less successful, however. Their equations can be solved explicitly only for very specific situations and they require experimental input in terms of both the measured cell volume
changes and changes in the mitotic index. The model itself is also not very intuitive and is difficult to manipulate.

Kubitschek has developed similar models and experimental data for bacteria. He concludes that the volume increase is linear with time and that furthermore this behavior is general for all cells (2, 3, 63). Much controversy has developed over Kubitschek's findings, centered not around experimental technique but around methods of interpreting the data (64, 65). Kubitschek has based many of his conclusions about the linear volume increase of cells during the cycle on the fine structure of the asynchronous volume distribution. Although the studies presented in Chapter I of this thesis disagree with Kubitschek's findings, many discussions with him over the past four years have been very helpful in developing the general techniques of cell volume spectroscopy used here.

Other purely theoretical models of cell population have been quite varied. Nooney (66) and Rubinow (22) have separately presented analyses of the distribution of cell ages in log phase populations, a point which is of key importance to all of these theories. Two other papers have also presented models of the behavior of synchronized cell populations (67, 68). At the present time no single model of cell populations has come to prominence as more complete or more useful than the others, probably because none as yet successfully respond to the needs of the experimenter as well as to the predictions of the theorist.

In association with the use of electronic volume sizing methods considerable attention has been paid to the detailed
theory and technical problems of volume spectrometry by several authors. Hurley has presented a physical analysis of an 'ideal' aperture (69), and formally derived the linear relationship between cell volume and voltage pulse height. Grover et al. have analyzed the electrical and hydrodynamic properties of the non-ideal volume sizing orifice and compared theory with experimental data obtained from rigid spheres (70, 71). A good degree of precision was found to be obtainable if conditions are properly controlled. Kubitschek has pointed out other sources of possible distortion, especially the shape and length of the aperture (72).

Cell volume, though it usually shows a good correlation with cell age and position within the cell cycle, can in many instances be uncoupled from its relationship to cell age. Rosenberg and Gregg in a particularly useful experimental investigation have shown that many external agents can greatly change cell volume kinetics (73). The effect of many of these agents and other biological parameters within the cell cycle can thus be better understood. Shank and Burki have reported cell volume to be associated with cell surface charge (74). However, since their method of synchrony using chemical blocking is believed to alter cell volume (73), the results cannot be simply related to those from mitotic selection synchrony experiments.

An important study by Fox and Pardee demonstrated that the cell volume immediately after mitosis for a given cell does not correlate with the length of the following G1 phase for that cell (60). Kimball et al. report simultaneous measurements and correlations between: (i) cell size, (ii) DNA content, and
(iii) protein content, using the combined techniques of electronic sizing, microphotometry and autoradiography (75). Studies such as Kimball's, correlating multiple cell parameters, can be expected to be particularly valuable as a future means of investigating the interrelationships between cellular processes. All of these investigations demonstrate that the one-to-one correspondence between cell volume and cell age that exists in synchronized cultures derived from mitotic selection is easily disturbed by external factors. This alerts one to the dangers of physical and chemical methods of synchrony in studying cell volume. A general review of the different procedures used for synchronization of mammalian cells, and the possible artifactual effects of these methods on experimental studies, has been presented by Nias and Fox (76).

The study of mitochondrial dynamics by electron microscopy described in Chapter II raises some interesting questions concerning the interpretation of three dimensional structures from two dimensional cross sections. An article by Elias discusses the various problems that might occur (77). Havelkova and Necas have considered specifically the problem of the evaluation of mitochondrial structure and shape from thin sections, and have provided information which shows this to be a minor experimental problem in the type of study presented in Chapter II (78, 79). Løvlie investigated the dynamics of another organelle--the chloroplasts, in the alga Ulva mutabilis (80). He showed a linear increase in organelle function (respiration) during the first portion of the division cycle,
with constant or decreasing function towards the end of the cycle. This system cleverly avoids many of the problems of interpretation due to uncertainty in organelle number since this alga has only a single chloroplast. Unfortunately no mammalian counterpart is known.

Cellular and subcellular dynamics have also been studied in vivo and although these experiments are usually less successful at providing quantitative results, they add another dimension to the literature. Epifanova and Terskikh's review (59) of the resting periods in the cell life cycle and a paper by Burns and Tannock on the existence of a G0 phase (81) present the evidence for using a cell cycle model in populations which are not rapidly dividing. Young and DeVita (82) and Gavosta and Pileri (83) discuss the cell cycle characteristics of human solid tumors, while Rajewsky (84) and Cikes (85) present similar experimental information for the kinetics of transplantable animal tumors.

Though studies of cell cycle dynamics are generally directed at increasing our fundamental knowledge about the cell, some practical application including that to cancer therapy has already been made. Frindel and Tubiana have excellently reviewed the radiobiology of the cell cycle and pointed out applications in radiotherapy (86). Steward and Hahn building on such data have presented arguments for the application of age response functions to the optimization of treatment schedules (87). A discussion of some of the experimental findings presented in Chapter III in this context has been presented at a radiotherapy conference by W. K. Sinclair (88).
The problem of cell growth and division continues as one of the fundamental areas of biological research. Much enlightenment as to the processes that occur during cell replication, and their interactions in time, has already resulted from past research of this kind. Our description of the cell is no longer a static, but rather a dynamic one. It spans a level ranging from the behavior of individual atoms to that of whole cell populations. The gaps between macromolecular structures and subcellular organelle function are beginning to be filled in, and the running of the whole machine -- the cell-- becomes more understandable. All of the diverse processes that lead to the living nature of cell growth and division are complex, but singular in their goal of cellular growth and proliferation. The desire to understand these processes in detail is strong.
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   the applicability of the Collins-Richmond principle. 
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### TABLE 1:

**COMPOSITION OF EM-15 GROWTH MEDIUM**

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/liter, without serum</th>
</tr>
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<tr>
<td>NaCl</td>
<td>7400.0</td>
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<tr>
<td>KCl</td>
<td>285.0</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
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</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>154.0</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$·7H$_2$O</td>
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<tr>
<td>KH$_2$PO$_4$</td>
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</tr>
<tr>
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<tr>
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</tr>
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<td>L-Cystine</td>
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<tr>
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<tr>
<td>L-Leucine</td>
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<tr>
<td>L-Lysine</td>
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<tr>
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<td>100,000 units/L</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100,000 mcg/L</td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td>14.6% v/v*</td>
</tr>
</tbody>
</table>

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*In a "batch" of medium as prepared at ANL, the total volume of ingredients is 2983 ml (2941 ml H$_2$O + 41 ml other ingredients) before serum is added. From this 75 ml is removed for test purposes and 500 ml of serum added, i.e. 14.6% v/v.*
TABLE 2:
WHOLE CELL AND ORGANELLE VOLUMES FOR
ASYNCHRONOUS CHINESE HAMSTER CELLS

mean whole cell volume = 710 \( \mu m^3 \) \( \pm \) 25 (experiment #G-2)

<table>
<thead>
<tr>
<th></th>
<th>Direct Expt. #G-43</th>
<th>Indirect Expt. #G-45</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean nuclear volume</td>
<td>194 ( \mu m^3 ) ( \pm ) 25</td>
<td>188 ( \mu m^3 ) ( \pm ) 20</td>
</tr>
<tr>
<td>total mitochondrial volume</td>
<td>32 ( \mu m^3 ) ( \pm ) 7</td>
<td>40 ( \mu m^3 ) ( \pm ) 5</td>
</tr>
<tr>
<td>mean number of mitochondria/cell</td>
<td>58 ( \pm ) 15</td>
<td>111 ( \pm ) 20</td>
</tr>
<tr>
<td>mean volume per mitochondrion</td>
<td>0.55 ( \mu m^3 ) ( \pm ) 0.20</td>
<td>0.36 ( \mu m^3 ) ( \pm ) 0.12</td>
</tr>
<tr>
<td>Medium</td>
<td>$N_1 \times 10^5$</td>
<td>$T_1$</td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
<td>-------</td>
</tr>
<tr>
<td>EM-15</td>
<td>1.45</td>
<td>20</td>
</tr>
<tr>
<td>HU-15</td>
<td>1.55</td>
<td>20</td>
</tr>
<tr>
<td>EMT-15</td>
<td>1.52</td>
<td>20</td>
</tr>
<tr>
<td>HUT-15</td>
<td>1.60</td>
<td>20</td>
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</tbody>
</table>
TABLE 4:
NUTRITIVE CAPACITY AND POPULATION DOUBLING TIME
MEAN VALUES AND STANDARD DEVIATIONS

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of Experiments</th>
<th>$t_d$ (hr)</th>
<th>$N_c \times 10^6$ (hr/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM-15</td>
<td>7</td>
<td>10.4 ± 1.1</td>
<td>6.8 ± 2.3</td>
</tr>
<tr>
<td>MEM-15</td>
<td>6</td>
<td>10.2 ± 1.6</td>
<td>10.4 ± 4.5</td>
</tr>
<tr>
<td>HUT-15</td>
<td>7</td>
<td>10.0 ± 2.3</td>
<td>9.8 ± 0.7</td>
</tr>
</tbody>
</table>
Figure 1: Population parameters as a function of time for an initially synchronized population: 2 1/2 cycles of a cell line with an average generation time $T_0 = 9.5$ hours. (a) Number of cells vs time, (b) Cell volume vs. time assuming linear volume growth. Note increasing 'rounded' effect due to decay of synchrony.
RELATIVE VOLUME, channel number

VOLUME DISTRIBUTION
- Coulter Analyzer - 500,000 cells
- Photographic Analysis - 700 cells

NUMBER OF CELLS, photographic method

NUMBER OF CELLS, electronic method

VOLUME, $\mu^3$

XBL 725-164
Figure 3: Comparison of experimental volume distributions for an asynchronous population of Chinese hamster cells V79-S171. The histogram is from a photographic analysis. The solid curve is the electronic volume spectrum obtained at Argonne. The dotted curve is volume spectrum obtained at Berkeley.
Figure 2: Comparison of experimental volume distributions for an asynchronous population of Chinese hamster cells V79-S171. The histogram is from a photographic analysis of 700 cells. The smooth curve is the volume spectrum measured electronically by Coulter aperture and multichannel analyzer; 500,000 cells.
Figure 4: Typical volume distributions for synchronous Chinese hamster cells V79-S171; six distributions cover two cycles of division ($T_0 = 9.5$ hours). Experimental values of the peak volumes of the distribution are denoted by arrows; dotted curves depict separation of distributions into parent and daughter cell subgroups at times when the whole population is dividing. Channel numbers correspond linearly to cell volume.
Figure 5: Cell volume (peak) vs. time for synchronous cultures of Chinese hamster cells V79-S171. The smooth curves are theoretical computations for linear and exponential growth models, equations (5) and (6), Chapter I, choosing, as indicated by the experimental data, an average generation time $T_0 = 9.5$ hours and with a coefficient of variation $CV = 9.5\%$ on the first cycle; and $T_0 = 9.0$ hours (due to a shortening of $G_1$) in the second cycle. Approximate subdivisions of the cell cycle as defined by DNA synthesis are shown above the same axis.

• starting population.
• starting population, undivided cells.
• cells during first cycle after synchronization.
• cells during second cycle after synchronization.
△ cells, both divided and undivided, at the end of the first full cycle after synchronization, during division.
□ cells during the third cycle after synchronization.
△ cells, divided and undivided, at the end of the second full cycle after synchronization.
Figure 6: Cell volume (peak) vs. time for synchronous cultures of Chinese hamster cells, V79-325. The smooth curves are based on theoretical calculations for linear and exponential growth, equations (5) and (6), Chapter I, choosing, as indicated by the experimental data, an average generation time $T_o = 15.3$ hours with CV = 6.5%. Approximate subdivisions of the cell cycle as defined by DNA synthesis are shown above the time axis.
Figure 7: The relative volume dispersion (measured by the half-width of the distribution at halfheight, \( \Delta v/v \)) vs. time for synchronous cultures of Chinese hamster cells: (a) V79-S171 and (b) V79-325. The symbols for the experimental points change after each cell division. Arrows at right indicate values for the asynchronous populations of these cell lines. Note: better synchrony is seen in (b).
Figure 8: A photomicrograph of a field from an autoradiograph of Chinese hamster V79-S171 cells labeled with a 15 min. pulse of $^{3}$HTdR and stained with iron hematoxylin after developing (~5000X). Most cells shown are labeled with many grains except for several evident mitotic figures. Note especially the centrally located prophase cell and an off center telophase cell demonstrating a chromosome bridge.
Figure 9: An electron micrograph of an 800Å thick central cross section through a single Chinese hamster V79-S171 cell printed at a final magnification of 18,500X.
Figure 10a. Percent labeled cells by tritiated thymidine incorporation, and 10b. mean cell volume (relative units and cubic microns) vs. time for synchronized Chinese hamster V79-S171 cells. Points are experimental results with error bars denoting the Poisson statistical uncertainty of one standard deviation (eq. (1)). Solid curves drawn through the data points are the predictions of a computer model for the synchronized population (Sinclair and Ross, 1969).
Figure 11a. Nuclear area fraction, $N/(N+M+C)$; b. Mitochondrial area fraction, $M/(N+M+C)$; and c. Mitochondrial number density $M_N/(N+M+C)$, vs. time for synchronized Chinese hamster V79-S171 cells--same experiment as Figure 10. Error bars about the experimental points denote Poisson statistical uncertainty of one standard deviation derived from counting individual units of area. Solid curves were drawn by hand.
Figure 12a.  Mean nuclear volume, $\mu^3$; b) Mean total mitochondrial volume, $\mu^3$; and c) Average number of mitochondria per cell vs. time for synchronized Chinese hamster V79-S171 cells. Error bars about the experimental points denote Poisson statistical uncertainty of one standard deviation derived from counting individual units of area. Solid curves were drawn by hand.
Figure 13: Growth curves, cell number versus time, of Chinese hamster V79-S171 cells grown in four variations of EM-15 medium from an experiment performed by W. K. Sinclair: a) EM-15, X; b) EM-15 + 2.5% v/v NCTC-109 = HU-15, ; c) EM-15 + 2% v/v 0.03% trypsin = EMT-15, Δ; d) EM-15 + 2.5% v/v NCTC-109 + 2% v/v 0.03% trypsin + HUT-15, 0. Points with centering lines indicate the transition from log to stationary phase for each curve.
Figure 14: Nutritive capacity in units of $10^6$ cell-hours/ml (left axis), and cell population doubling time in hours (right axis), for Chinese hamster V79-S171 cells grown in MEM medium, versus percent fetal calf serum supplement. Circles and solid line for nutritive capacity, $N_c$, squares with dotted line for cell population doubling times, $t_d$. 
Figure 15: Average cell volume (relative scale) vs. time for Chinese hamster cells, V79-S171, in HUT-15 (circles and solid line).  a) Changes from log to stationary phase; b) Changes for cells in stationary phase resuming growth in fresh medium.
Figure 16: A summary of the changes in cell population parameters as cells progress from log phase to early, mid and late stationary phase for Chinese hamster cells grown in two media: a) HUT-15; b) EM-15. Percent cells synthesizing DNA, RNA and protein determined by uptake of labeled precursors; percent viable cells determined by plating efficiency. A schematic diagram of the distribution of the cell population within the compartments of the cell cycle for each phase is also shown. The cell cycle distribution histogram shows relative percent of the population in $M$, $G_1$, $S$, $G_2$. 
### (A) HUT-15

<table>
<thead>
<tr>
<th>PHASE</th>
<th>% CELLS SYNTHESIZING PLATING % EFFICIENCY</th>
<th>CELL CYCLE DISTRIBUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% DNA</td>
<td>% RNA</td>
</tr>
<tr>
<td>Log Phase</td>
<td>56±5</td>
<td>80±10</td>
</tr>
<tr>
<td>Early</td>
<td>33±3</td>
<td>-</td>
</tr>
<tr>
<td>Mid</td>
<td>10±2</td>
<td>25±10</td>
</tr>
<tr>
<td>Late</td>
<td>1±1</td>
<td>-</td>
</tr>
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</table>

### (B) EM-15

<table>
<thead>
<tr>
<th>PHASE</th>
<th>% CELLS SYNTHESIZING PLATING % EFFICIENCY</th>
<th>CELL CYCLE DISTRIBUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% DNA</td>
<td>% RNA</td>
</tr>
<tr>
<td>Log Phase</td>
<td>72±5</td>
<td>90±10</td>
</tr>
<tr>
<td>Early</td>
<td>52±5</td>
<td>-</td>
</tr>
<tr>
<td>Mid</td>
<td>38±3</td>
<td>60±10</td>
</tr>
<tr>
<td>Late</td>
<td>35±3</td>
<td>-</td>
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</tbody>
</table>
Figure 17: A growth resumption experiment for Chinese hamster cells in mid stationary phase diluted into fresh medium. Circles and solid line for HUT-15 medium; squares and dotted line for EM-15. a) Percent labeled cells, b) cumulative mitotic index, and c) instantaneous mitotic index versus time. Error bars indicate Poisson uncertainty in counting.
The graph shows the percentage of labeled, cumulative mitotic, and mitotic indices over time (0 to 30 hours). The data is represented with error bars for each time point.
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